Glutamate Dehydrogenase and 2-Oxoglutarate Reductase Electrophoretic Patterns and Deoxyribonucleic Acid-Deoxyribonucleic Acid Hybridization among Human Oral Isolates of Fusobacterium nucleatum

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The electrophoretic mobilities of glutamate dehydrogenase and 2-oxoglutarate reductase were compared for three reference strains and 30 human, oral isolates of Fusobacterium nucleatum. Both enzymes allowed the same strains to be grouped into three electrophoretic clusters, designated groups Fn-1, Fn-2, and Fn-3. Group Fn-1 contained the type strain of F. nucleatum, strain ATCC 25586, and nine clinical isolates. Group Fn-2 comprised 20 strains and appeared to contain the strains of F. nucleatum that are isolated most commonly from oral cavities. Strain NCTC 10953 (formerly "Fusobacterium polymorphum") was a member of this cluster. Strains of group Fn-3 were rarely isolated; this group contained three isolates and reference strain NCTC 11362, which was listed previously as "Fusobacterium fusiforme." The deoxyribonucleic acid (DNA) base compositions of all strains were between 25 and 27 mol% guanine plus cytosine. Under optimal conditions of DNA-DNA hybridization, all of the strains exhibited high levels of DNA homology (73 to 99%) to the three reference strains and 30 human, oral isolates of F. nucleatum. Strain NCTC 11362 (formerly "Fusobacterium fusiforme"), as well as 30 clinical isolates from normal and diseased sites of human oral cavities.

Cultivation and identification. All of the strains were maintained by weekly subculture on 2.5% (vol/vol) blood agar plates in an atmosphere containing 10% (vol/vol) CO₂ and 5% (vol/vol) H₂ in N₂ at 37°C. Each strain was inoculated onto freshly prepared Columbia agar (Oxoid Ltd., London, England) supplemented with 6% horse blood and 0.1% vitamin K and was incubated anaerobically for 48 h. A bacterial suspension corresponding to a McFarland no. 4 density standard was used to inoculate a RapID ANA Kit (API System, La Balme-Les-Grottes, France), and the strips were incubated aerobically at 37°C for 4 h. Strains were identified as described by Murdoch et al. (17). The method used for acid end product analysis for culture supernatants has been described previously (8).

Preparation of cell-free extracts and enzyme electrophoresis. Cells were harvested from 2-day-old blood agar cultures and were suspended in 0.5 ml of 0.1 M tris(hydroxymethyl)amino-methane hydrochloride buffer (pH 8.5). Each cell suspension was shaken with Ballotini beads (grade 12) for 10 min in a Mickle tissue disintegrator at 4°C. The suspension was centrifuged at 37,000 × g for 10 min, and the supernatants were electrophoresed as described previously (20). Electrophoresis was for 90 min at 15 V/cm of gel in 0.04 M barbitone-acetic acid buffer (pH 8.6). GDH was visualized by using a solution of glutamate (20 mg/ml), nicotinamide adenine dinucleotide (5 mg/ml), phenazine methosulfate (0.04 mg/ml), and thiazolyl blue tetrazolium (0.02 mg/ml) in 0.1 M tris(hydroxymethyl)aminomethane hydrochloride buffer (pH 9.0). OGR was visualized by replacing the glutamate with a 2-hydroxyglutarate solution (10 mg/ml).

Isolation, purification, and base composition determination of DNA. DNAs from all strains were prepared as described previously (4). The DNA base composition was estimated from the melting temperature of the DNA in saline citrate (0.15 M NaCl plus 0.015 M trisodium citrate, pH 7.0) by

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preparations were dialyzed in deionized water for 48 h before labeling. For hybridization experiments, 3 μg of each reference DNA was labeled in vitro with deoxy[1',2',5-3H]cytidine 5'-triphosphate by nick translation (10), using a commercial nick translation kit (Amersham Corp., Arlington Heights, Ill.). The reaction was terminated after 40 min, and the DNA was separated from unincorporated nucleotides by gel exclusion chromatography through Sephadex G-50 and dialyzed in 0.42 M NaCl. The DNA was sheared by sonication for 3 min with a 3-mm probe at a power setting of 7 (3 to 4 A for three 10-s bursts; Soniprobe; Dawes Instruments, Ltd., London, England).

**DNA-DNA hybridization.** DNA homology experiments were performed as described previously (19), with minor modifications. The unlabeled DNAs to be used for all hybridizations with the labeled probe DNA (from the three reference DNA preparations) were dialyzed in 0.42 M NaCl and sheared as described above to give a fragment size of approximately 500 base pairs. A reaction mixture containing 0.02 μg of the labeled reference DNA and 20 μg of unlabeled DNA was mixed in 200 μl of 0.42 M NaCl, and 200 μl of light paraffin oil was layered on top. The mixture was denatured for 10 min at 100°C, and hybridization was allowed to proceed for 93 h at either 55°C (optimal hybridization) or 75°C (stringent conditions of hybridization). The tubes were then placed in an ice bath for 20 min to stop the reaction. S1 nuclease (100 U/ml; Sigma Chemical Co., Dorset, England) in a buffer containing 0.1 mM ZnSO4, 0.15 M NaCl, 30 mM sodium acetate (pH 4.5), and 20 μg of sheared and denatured calf thymus DNA (type V; Sigma) per ml was added to all tubes. The mixture was incubated for 20 min at 50°C, precipitated with 10% trichloroacetic acid, and filtered on membrane filters (Schleicher & Schuell, Dassel, Federal Republic of Germany). Cocktail T scintillation fluid (BDH, Essex, England) was added to the filters, which were counted with a model SL30 liquid scintillation counter (Intertechnique, Dover, N.J.). The level of hybridization was determined as a percentage of the value for the homologous reaction. The level of recovery of labeled DNA in the homologous reaction was 82%. A correction was made for self-reassociation of labeled DNA by using calf thymus DNA as a control.

**RESULTS AND DISCUSSION**

The three reference strains and 30 clinical isolates used in this study were readily identified as *F. nucleatum* by using the RapID ANA system (API System). In addition, the metabolic end products of these organisms corresponded to those reported previously (8). Care was taken to distinguish these bacteria from other oral species, such as *Fusobacterium sulci*, *Fusobacterium alocis*, and *Fusobacterium periodonticum*, by using previously described tests (2, 23). Among the 33 strains of *F. nucleatum* tested, three groups were clearly discernible on the basis of GDH electrophoretic patterns. Furthermore, the compositions of the three GDH clusters formed were supported by the OGR electrophoretic mobility patterns (Fig. 1). The mobility clusters were designated groups Fn-1 (strains which had GDH and OGR mobilities of 0.8 and 1.8 cm, respectively), Fn-2 (isolates which had GDH and OGR mobilities of 1.3 and 2.3 cm, respectively), and Fn-3 (strains which had GDH and OGR mobilities of 1.8 and 2.0 cm, respectively) (Fig. 1). The variation in GDH and OGR electrophoretic mobilities within groups was minimal (ca. 1 to 2 mm), and the patterns, which were tested on several occasions, were consistent. Group Fn-1 comprised 10 strains and included *F. nucleatum* ATCC 25586.T. Isolates which belonged to this group were readily identified by their distinctive morphology on blood agar plates, typical of that associated with *F. nucleatum*. After overnight growth, colonies were round, convex, and 0.5 to 1.0 mm in diameter and had a greyish white appearance. Group Fn-2 strains appeared to be the most frequently isolated organisms from human oral cavities. This group contained the previously described species “*F. polymorphum*” and consisted of 20 identical strains. After overnight culture, colonies on blood agar plates were characteristically round, convex, 1 to 2 mm in diameter, smooth, glistening, and entire. If cultures were incubated for more than 3 days, they became indistinguishable from group Fn-1 isolates. Group Fn-3 strains were the least commonly isolated strains of *F. nucleatum*. However, the four strains which belonged to this group were recognized by their distinctive colonial morphology; this group contained the previously described organism “*F. fusiforme*” (5, 6) NCTC 11362 (National Collection of Type Cultures, Central Public Health Laboratory, London, England). Colonies on blood agar plates were 1 to 3 mm in diameter and irregular and had a low cone shape with speckled surfaces, and pitting was frequently observed if the colonies were dislodged.

Despite the clear subdivision of *F. nucleatum* strains into three electrophoretic subgroups, DNA base composition determinations and DNA-DNA hybridization experiments indicated that human isolates of *F. nucleatum* belong to the same species. The guanine-plus-cytosine values ranged be-
tween 25 and 27 mol% (Table 1), well within the limits of a clearly defined species. DNA-DNA hybridization experiments carried out against three reference DNA probes belonging to groups Fn-1, Fn-2, and Fn-3 confirmed the relatedness of these strains. Initially, DNA-DNA reassociation experiments were performed under optimal conditions (55°C). The levels of DNA homology were high with each probe and ranged between 73 and 99% (Table 1). Under more stringent conditions, these values ranged between 25 and 27 mol% (Table 1), well within the limits of a species. However, several substitutions may also take place without affecting the electrophoretic properties of an enzyme (N. Dance and D. C. Watts, Biochem. J. 84:114, 1962). Differences in electrophoretic migration patterns of enzymes may result from minor genetic alterations and may not reflect taxonomic identity, as in the present study. Often such differences reflect genetic heterogeneity (18, 20, 21) and can be used to identify species which are otherwise difficult to differentiate. Since the electrophoretic patterns of GDH can be used to identify species which are otherwise difficult to differentiate, these organisms to other members of the genus

Variations in electrophoretic mobilities occur due to differences in the net changes between enzyme molecules (22). A single amino acid substitution may alter the surface properties of a molecule and affect its electrophoretic mobility. However, several substitutions may also take place without affecting the electrophoretic properties of an enzyme (N. Dance and D. C. Watts, Biochem. J. 84:114, 1962). Differences in electrophoretic migration patterns of enzymes may result from minor genetic alterations and may not reflect taxonomic identity, as in the present study. Often such differences reflect genetic heterogeneity (18, 20, 21) and can be used to identify species which are otherwise difficult to differentiate. Since the electrophoretic patterns of GDH can be used to identify species which are otherwise difficult to differentiate, these organisms to other members of the genus.
and avirulent strains exist. Although most chemotaxonomic data indicate that F. nucleatum is a homogeneous species (9, 14, 26), several studies of oral isolates to determine soluble protein or polypeptide patterns have indicated that there is considerable heterogeneity (1, 2, 13). However, the electrophoretic patterns derived were too cumbersome and varied for clustering of isolates (1).

As GDH consistently had a distinctive migrating position within each group, strains could be readily assigned to one of the three clusters by using this enzyme pattern. However, the combined use of GDH and OGR may be helpful. To understand the ecology of F. nucleatum in its natural ecosystem, it may be necessary to recognize the diversity within the species. Tests used so far either have obscured this diversity or have been too complex to assess. The method which we describe provides a simple and reliable technique for studying the ecology of F. nucleatum.

ACKNOWLEDGMENT

S.E.G. acknowledges an ORS award for this work.

LITERATURE CITED